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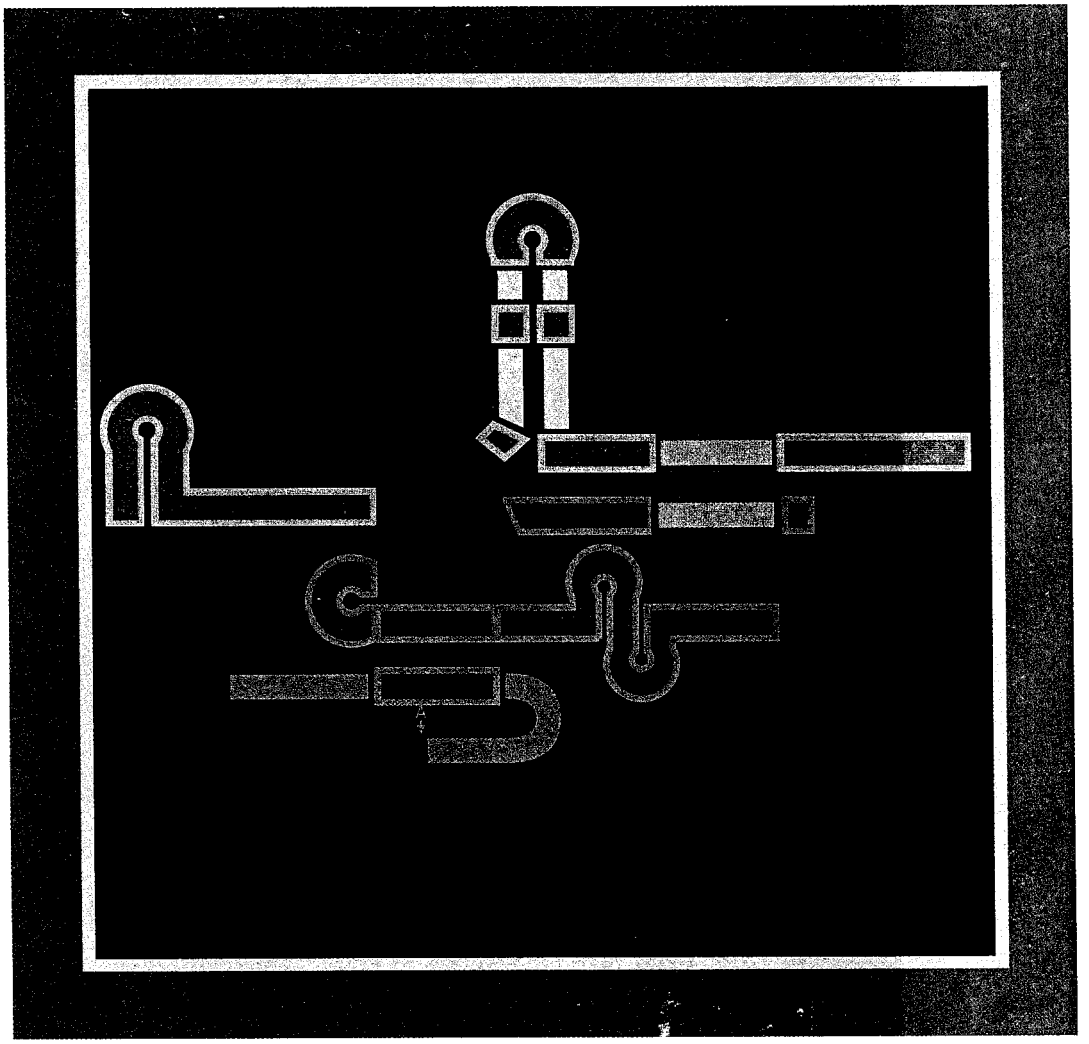

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MOLECULAR AND GENETIC APPROACHES TO RETROVIRUSES AND ONCOGENES

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In this laboratory, retroviruses have traditionally served as points of departure for studying various aspects of eukaryotic cells at the molecular level. Our attempts to understand how retroviruses multiply within infected cells and how retroviruses cause cancers have led to work on a variety of topics, including cell surface receptors, DNA recombination, translational control, inter- and intracellular signaling, hematopoiesis, embryonic development, and mammary carcinogenesis.

1. **Early events in virus infection require entry into cells through host-encoded receptors; we have recently cloned avian genes encoding receptors for a subgroup of Rous sarcoma virus (RSV) and shown that the receptors resemble the receptor for low-density lipoproteins (LDL).**

2. **After viral DNA is synthesized by reverse transcriptase, it is efficiently inserted into chromosomes by a small viral protein, integrase, that performs a recombination reaction required for transposition of many types of elements. Recent studies of retroviral integrases identify some of the residues essential for their biochemical activities and the structural determinants that influence choice of integration sites in DNA and chromatin.**

3. **Synthesis of reverse transcriptase and integrase usually requires efficient shifting of reading frame by ribosomes translating viral RNA, in response to short sequences and RNA structural elements, commonly pseudoknots; the determinants of frameshifting**

efficiency can now be studied with genetic, structural, and biochemical methods in yeast and mammalian systems.

4. **Most of the highly tumorigenic retroviruses carry oncogenes, derived from host proto-oncogenes; several of these belong to a gene family (*src*) that encodes cytoplasmic protein-tyrosine kinases implicated in differentiated functions; targeted mutations of some of these genes (*hck* and *fgr*) have been made in the mouse germ line to determine their physiological roles in hematopoiesis. In efforts to understand the actions of src protein, the protein has been localized to endosomes in fibroblasts, and extracatalytic regions that mediate interactions with other proteins have been defined with genetic and biochemical means.**

5. **Most weakly tumorigenic retroviruses activate cellular proto-oncogenes by insertion mutations; the *Wnt-1* gene, discovered as a target for insertional activation by the mouse mammary tumor virus, belongs to a large family of genes encoding secretory proteins involved in important developmental events in many organisms. In attempts to identify cell-surface receptors for Wnt proteins, we have developed several bioassays for *Wnt* genes and learned to make cell-free Wnt protein in a complex with the surface antigen of hepatitis B virus. Site-directed mutants of *Wnt-1* include temperature-sensitive alleles and alleles encoding active, transmembrane proteins. Two *Wnt* genes have been characterized in *C. elegans*. Finally, a *Wnt-1* transgenic model for mammary carcinogenesis has been used to identify additional genetic components of a multistep neoplastic process.**

THE RETROVIRAL LIFE CYCLE

The study of retrovirus replication has been instructive about eukaryotic cells in many ways during the past twenty years: Retroviruses provided the first source of reverse transcriptase, their proviruses were the first examples of precise recombination products in eukaryotic DNA, and their strategies for gene expression include transcriptional enhancers, glucocorticoid responsiveness, alternative splicing, ribosomal frameshifting, nonsense suppression, and intricate protein-nucleic acid interactions during virus assembly. The need to understand these and other aspects of retroviruses has become more urgent with the discovery that AIDS is caused by a retrovirus, the human immunodeficiency virus (HIV).

Our current work on the retroviral life cycle is focused upon three events: 1) the entry of virus into cells via host-encoded receptors; 2) the integration of viral DNA into host chromosomes; and 3) the synthesis of polymerase gene products by ribosomal frameshifting. Although we continue to perform some of these studies with avian and murine retroviruses, we give increasing attention to HIV.

Retroviral Receptors

Entry of retroviruses into cells depends upon host-encoded transmembrane proteins that serve as receptors for viral envelope glycoproteins. The remarkable specificity of virus-host interactions has been known for over twenty years from studies of the polymorphic envelope proteins of avian retroviruses, yet little biochemical information is available about the receptors or about the nature of their interactions with viral envelope glycoproteins. Over the past few years, receptors for several animal viruses have been identified as members of the superfamily of immunoglobin genes; perhaps the most important example is the lymphocyte cell-surface antigen CD4, a simple transmembrane glycoprotein that is required for attachment of HIV to target cells. Recently, the receptor for ecotropic murine leukemia virus (MLV) was shown to be a very different type of protein, an amino acid permease with fourteen transmembrane domains.

Paul Bates and John Young have recently cloned the gene encoding the receptor for one of the five subgroups of RSV. This was accomplished by a gene transfer method in which chicken DNA was introduced into mammalian cells, which lack functional receptors; target cells that acquired a receptor gene were identified by infection with subgroup A avian virus vectors carrying a selectable marker. Although cDNA clones have been difficult to obtain, we have deduced the coding sequence of the gene by exon trapping in a retroviral vector. The results were surprising: The putative receptor is a very small protein, just over 100 amino acids in length, with a single transmembrane domain and an ectodomain that

closely resembles a portion of the binding region of the low-density lipoprotein (LDL) receptor. In addition, the gene appears to be differentially spliced, generating at least two membrane-bound proteins (one of which has been shown by Hao Wang to be GPI linked) and a secreted protein. Antisera against the ectodomain have been used by Paul and David Chu (an MSTP student on rotation) to show that the gene produces a cell surface glycoprotein in transfected cells and that virus infection of normal cells can be blocked immunologically. Hao Wang is now trying to assess the normal composition and function of the receptor, to ask (with Judy White's laboratory) whether it can bind directly to viral envelope protein, and to examine its structure (in collaboration with Dave Agard's laboratory). (Some of these studies continue to be pursued with John Young, now on the UCSF faculty at the Gladstone Institute for Virology and Immunology.)

Mike Lochrie is using methods similar to those employed with the avian virus receptor to clone receptors for feline leukemia viruses and to analyze a known receptor for subgroup B feline viruses. In addition, Richard Sutton, in collaboration with Dan Littman, has begun to try to clone a receptor for the human T cell leukemia virus (HTLV).

Proviral Integration

Like many transposable elements from plants, bacteria, yeast, and insects, retroviral proviruses can be found at many different sites in host genomes, but are always joined to host DNA at the same sites in viral DNA. The provirus contains viral genes arranged as they are in viral RNA (most commonly: *5'-gag-pol-env-3'*), flanked by long terminal repeats (LTRs) that are generated during reverse transcription and used for regulation of transcription. The LTRs terminate with short inverted repeats that form part of the att site required for integration, and the entire provirus is flanked by short direct repeats of cellular origin generated during the integration step. The only viral protein known to be required specifically for integration, the IN protein, is encoded by the 3' end of the *pol* gene.

Current studies of retroviral integration depend largely upon reconstruction of the reaction under *in vitro* conditions. This was first accomplished with viral nucleoprotein complexes from cells recently infected with MLV. It is now possible to study the reaction using only purified IN protein (usually prepared here from yeast expression systems) and labeled oligonucleotides representing att sites and targets. It is now known that two independent events are mediated by IN protein: 1) removal of two nucleotides from the 3' end of each strand in the linear form of viral DNA, and 2) staggered cutting of the target DNA, with a concerted, ATP-independent joining of newly created 5' ends to the viral 3' ends.

Andy Leavitt has isolated the HIV-1 IN protein from a yeast expression system in order to study the att site, target site, and IN sequence determinants of the steps in the integration reaction. He has shown that both steps in the reaction require a highly conserved CA dinucleotide at the site of 3' processing; that additional internal sequences confer specificity for the HIV IN protein; and that the selection of integration sites is dependent upon the sequence of the target oligonucleotide. In conjunction with Lily Shiue, who has made site-directed mutants of HIV-1 IN, several amino acid residues crucial for biochemical function have been identified. Some of these mutant genes have also been tested in an HIV-based virus vector system. Mike Chastain is now constructing HIV-MLV chimeric IN proteins in efforts to map functional domains. In addition, HIV-1 IN protein has been purified on a large scale to attempt X-ray crystallography in collaboration with Bob Rose in the Stroud laboratory.

To study the integration reaction in a fashion that more closely resembles what occurs in infected cells, Peter Pryciak has shown that MLV nucleoprotein complexes mediate integration *in vitro* into target DNA assembled into nucleosomes. Through the use of minichromosomes in which nucleosomes are accurately positioned, he has been able to map integration sites in relation to nucleosomes. This was done initially by cloning and sequencing reaction products, but the analysis has been remarkably simplified and enhanced by Peter's development of a polymerase chain reaction (PCR)-based assay that scores the position and frequency of integration events within several hundred nucleotides of a PCR primer site. The findings reveal that integration occurs more efficiently into DNA packaged nucleosomes than into nucleosome-free regions of DNA; that the sites most available for integration are in the major groove of DNA facing away from the nucleosomal core (thus occurring with a periodicity of about 10 bp, one turn of the DNA helix); and that nucleotide sequence also influences the selection of integration site. DNA-binding proteins, such as the yeast alpha-2 protein, block integration, creating a "footprint" in the PCR-based assay.

These results imply that the retroviral integration machine is sensitive to the status of eukaryotic chromosomes and may be able to survey their functional and structural properties *in vivo*, as well as *in vitro*. Peter and Hans-Peter Müller have shown that co-infection of cells with MLV and simian virus 40 (SV40, a virus with a small circular DNA genome) allows integration of MLV DNA into the many copies of SV40 minichromosomes in a strikingly nonrandom pattern. Hans-Peter is using various

strategies to bend DNA targets and thereby determine how site preference occurs. For example, when DNA is bent by contact with bacterial CAP protein or by bringing together ends of short linear duplexes, the distribution of integration sites is altered and resembles patterns observed with chromatin.

Ribosomal Frameshifting

All retroviruses synthesize reverse transcriptase and IN protein as *gag-pol* polyproteins that must be subsequently cleaved by a viral protease to form mature functional proteins. Yet most retroviruses encode *gag* and *pol* in different frames. Retroviruses solve this problem by directing ribosomes to slip back one nucleotide while decoding regions of *gag-pol* mRNAs in which the reading frames overlap. The slippage occurs at defined frequencies (generally 5-25%), in response to specific sequences at the frameshift sites (e.g., A AAU UUA or A AAA AAC), and requires secondary structural elements just downstream from the site. Similar frameshifting occurs during synthesis of a coronavirus RNA polymerase, the transposase of the prokaryotic element, IS1, and a subunit of *E. coli* DNA polymerase III.

The nature and function of RNA secondary structures downstream of frameshift sites continue to be important and incompletely resolved issues. Mario Chamorro, in conjunction with Neil Parkin, has precisely defined an RNA pseudoknot required for high-frequency frameshifting on mouse mammary tumor virus (MMTV) RNA, at the overlap of the *gag* and *protease* genes. Structural features of this and other pseudoknots are now being studied in collaboration with Ignacio Tinoco's laboratory at the University of California, Berkeley, using a combination of genetic, biochemical, and physical methods (including NMR). Preliminary results confirm the existence of the predicted pseudoknot. Moreover, substitution of a known pseudoknot for the MMTV pseudoknot indicates that not all pseudoknots are able to promote frameshifting. Neil and Mario have recently confirmed the importance of a hypothetical stem-loop downstream of the HIV-1 frameshift site; although the integrity of the stem-loop appears to have only minor effects upon frameshifting *in vitro*, it has a more dramatic effect when the *gag-pol* region of HIV-1 is expressed in cultured cells. Mario's study of the MMTV *gag-pro* frameshift site has also suggested that the efficiency of frameshifting is strongly influenced by the nature of the tRNAs decoding the frameshift site.

Susanna Lee has begun to study retroviral frameshifting in yeast, where genetic means can be used to identify tRNA's, other *trans*-active factors, and unsuspected *cis*-active elements that affect frameshift rates. By screening colonies for production of ß-galactosidase or selecting for high levels of CUP1 protein, Susanna has assembled a set of strains with variable levels of frameshifting in response to MMTV- and HIV-derived signals. Two complementation groups have been identified for cloning mutant genes have begun.

RETROVIRAL ONCOGENESIS AND PROTO-ONCOGENES

Retroviruses competent to induce tumors are conveniently grouped in two categories: 1) those highly oncogenic agents that carry oncogenes transduced from normal cells, and 2) those less efficient agents that lack their own oncogenes. Among the first group are viruses employing over twenty distinctive oncogenes (v-*onc*'s). Each v-*onc* is derived from a cellular proto-oncogene, and these in turn are often members of gene families. The second group of oncogenic retroviruses, those lacking their own oncogenes, includes several viruses producing a wide spectrum of diseases. Viruses of this second type usually act as insertional mutagens, enhancing the expression of adjacent cellular proto-oncogenes as an initial step in tumorigenesis. Some of these genes have also been transduced to form retroviral oncogenes, but many were discovered as novel targets for insertion mutation.

A surprising number of proto-oncogenes have proved to encode growth factors, growth factor receptors, components of cytoplasmic signaling networks, or transcription factors. Still, the biochemical steps in normal growth control remain incompletely defined, as are the mechanisms responsible for conversion of a normal cell to a cancer cell by activated oncogenes. It is increasingly evident that cancer results from a series of genetic events, in which inactivation of tumor suppressor genes is likely to be at least as important as activation of oncogenes. Furthermore, the role of proto-oncogenes in developmental events has become better appreciated, but it is complicated by the complementary functions of several members within gene families.

Our current studies of proto-oncogenes are largely confined to two gene families: 1) the *src* family, the first member of which was discovered as the cellular progenitor of the v-*src* oncogene of RSV, and 2) the *Wnt* family, the first member of which was found as the target for insertional mutation by MMTV.

The *src* Gene Family

v-*src* encodes a membrane-associated phosphoprotein of 60,000 daltons ($pp60^{v\text{-}src}$) that displays protein kinase activity *in vitro* and induces phosphorylation of tyrosine residues in several proteins *in*

vivo. The functionally significant target molecules have not been identified, however, and the physiological functions of the cellular progenitor, c-*src*, are not known. Mutations that augment the tyrosine kinase activity of pp60 appear to be required to convert c-*src* into an oncogene. Studies of c-*src* have been strongly influenced in the past few years by the recognition that it is one of about eight genes encoding very similar but differentially regulated proteins.

c-*src* is expressed in virtually all cells, often at high levels; its mRNA is differentially spliced in the nervous system; and c-src protein is phosphorylated by the cdc2-encoded kinase during mitosis. Nevertheless, as shown by Phil Soriano (Baylor), inactivation of the mouse c-*src* gene by targeted homologous recombination produces an unexpectedly limited phenotype: osteopetrosis (excessive bone deposition) resulting from defective osteoclast function. These findings suggest that *src* gene family members complement each other in many cell types, so that phenotypes may be best studied in specialized cells or revealed more generally after inactivation of combinations of such genes.

To these ends, in collaboration with Soriano's laboratory, Cliff Lowell has made targeted null mutations of the *hck* and c-*fgr* genes, two *src* gene family members expressed mainly in the hematopoietic lineage. Homozygous mice have been derived for these mutations, but, thus far, only subtle phenotypic changes have been found. Macrophages from *hck*-deficient mice phagocytize latex beads inefficiently, but even doubly homozygous *hck*$^-$ and *fgr*$^-$ animals appear overtly healthy and have apparently normal bone marrow and circulating blood cells. However, the doubly homozygous mice are unable to protect themselves against infection with *Listeria monocytogenes*. In addition, mice lacking both *src* and *hck* are anemic and leukopenic and lack splenic germinal centers. Maho Niwa is studying the basis of this hematopoietic phenotype.

Ken Kaplan has used immunofluorescent and cell fractionation methods to localize c-src protein in cultured fibroblasts. Although the product of v-*src* is mostly present in the plasma membrane, especially in adhesion plaques, p60$^{c\text{-}src}$ is associated mainly with endosomes, particularly in the region of the microtubule organizing center in interphase cells and near the spindle pole bodies during mitosis. Ken and Krissy Bibbins are introducing c-*src* mutants into *src*-deficient cells (supplied by Soriano) to study the determinants of the localization pattern and search for functional consequences of the association with endosomes. Preliminary findings suggest that *src* may have a role in maintenance of cell adhesion and that the kinase domain is neither necessary nor sufficient to direct src protein to focal adhesions.

Several years ago, we isolated a host-dependent mutant of v-*src*, a mutant that transformed chicken cells efficiently and mammalian cells poorly, and found a single amino acid change in the most highly conserved region of a non-catalytic domain, called SH2, that mediates interactions with phosphotyrosine and is found in many kinds of signaling proteins. As evidence accumulated for the importance of the SH2 domain, Hisamaro Hirai introduced many site-directed mutations into these regions of a c-*src* gene made active for transformation. Several of these mutants are also host-dependent, with greater transforming activity in either avian or mammalian cells.

Multiple approaches are being taken to exploit the properties of these mutants to probe the interactions between src proteins and other cellular proteins. John Murphy is seeking chicken genes and mouse cell mutants that restore the ability of host-dependent SH2 mutants to transform mouse cells. In addition, he is attempting to generate intragenic, second site mutants that restore competence to transform mouse cells and testing the idea that differences in protein-tyrosine phosphatases may account for the host range phenomena. Helene Boeuf and Krissy Bibbins have produced wild-type and mutant SH2 domains as fusion proteins in *E. coli*. They have used these proteins as affinity reagents with extracts of mammalian and avian cells and with phosphotyrosine peptides on beads. The results are largely consistent with the recently published structure of the src SH2 domain bound to phosphotyrosine, although at least one residue predicted to contact phosphotyrosine can be altered without apparent loss of binding activity. SH2 mutants, even those with strong host-dependence for transformation, bind phosphotyrosine-containing proteins in avian or mammalian cell extracts in accord with their ability to bind phosphotyrosine-containing peptides on beads. Thus, host-dependence remains unexplained, but an important phenomenon.

The *Wnt* Gene Family

The *Wnt-1* gene was discovered in 1982, using the technique known as "transposon tagging" to identify genes that serve as targets for insertion mutation during mammary tumor induction by MMTV. About 75% of tumors in C3H mice have MMTV insertion mutations that activate expression of *Wnt-1*. (In mammary tumors in some other mouse strains, additional genes have been isolated as targets for MMTV insertion mutations. The best-studied of these, the *int*-2 gene, is a member of the fibroblast growth factor gene family. Another, *int*-3, encodes a transmembrane receptor of unknown function.)

The *Drosophila* homolog of *Wnt-1* is the segment polarity gene, *wingless*. Furthermore, mammals

contain several genes closely related to *Wnt-1* and homologs have been cloned from a wide range of vertebrate and invertebrate species (see below). These genes have been strongly implicated in developmental events by their restricted patterns of expression, particularly in the embryo; by the consequences of null mutations (cerebellar and mid-brain malformations after targeted mutation of mouse *Wnt-1*, segmentation defects with *wingless* mutations); and by the induction of axis duplication after injection of *Wnt* mRNAs into the early frog embryo.

The nucleotide sequence of *Wnt-1* cDNA predicts a protein of 370 amino acids, with a signal peptide, a cysteine-rich carboxyterminus, and four N-linked glycosylation sites. Over the past few years, we have shown that the primary product is subject to multiple modifications in cultured cells, including proteolytic cleavage and several glycosylations, producing at least five distinguishable forms of Wnt-1 protein in the secretory pathway. At least two of these forms are secreted and associated with the extracellular matrix and the cell surface. Jan Kitajewski also found that Wnt-1 proteins are associated with a 78 Kd protein known as BiP, which binds to certain secretory proteins in the endoplasmic reticulum.

Unlike most other secretory proteins, Wnt proteins have not been obtained in a soluble, biologically active form. This fact has severely limited studies of the structure and function of Wnt proteins and may account for the failure to identify cell surface receptors for Wnt proteins. We have attempted to compensate for this problem by developing assays for *Wnt* genes in cultured cells. Although most cell lines do not appear to respond to the introduction of *Wnt* genes, we have found two that do: a mouse mammary epithelial cell line, called C57MG, that responds by dramatic morphological change and enhanced growth potential; and the rat pheochromocytoma line, PC12, that changes shape, becomes more adherent, and loses responsiveness to NGF.

In addition, because Wnt proteins are secreted and appear to act locally, we can assay genes in a paracrine manner by expressing them in a non responsive cell and observing changes in adjacent, non expressing responsive cells (C57MG).

Site-directed mutants of *Wnt-1* generated by John Mason and Jan Kitajewski have shown that the signal peptide is required for entry into the secretory pathway, association with BiP, and biological activity. Mutation of conserved cysteine residues results in loss of autocrine and paracrine activities. None of the glycosylation sites appears to be important for biological activity: *Wnt-1* is active in both autocrine and paracrine assays even when all four glycosylation sites have been mutated. Two mutants, one altering a cysteine residue and one a glycosylation site, are temperature-sensitive. Jan, Dave Leonardo (on a rotation project), and Neil Parkin have found that Wnt-1 chimeras with heterologous transmembrane domains can induce partial transformation of C57MG cells and axis duplication in *Xenopus* embryos. Karl Willert has been attempting to exploit some of these mutants, especially the ts mutants, to implicate biochemical mechanisms, such as tyrosine phosphorylation, in the Wnt signaling pathway. He has described the step in NGF-induced signaling that is blocked by Wnt-1 protein in PC12 cells; the *trk*-encoded NGF receptor is autophosphorylated in a normal fashion in *Wnt-1*-expressing PC12 cells, and other early consequences of NGF occur normally, but the cell fails to respond by production of neurite and neuronal markers.

Ian Taylor and Sophie Roy (a postdoctoral fellow in the Ganem laboratory) recently found that hepatoma cells co-transfected with vectors expressing Wnt-1 protein and hepatitis B surface antigen efficiently secrete particles that contained both proteins in immunoprecipitable form. Importantly, the particles exhibit biological activity. They induce morphological changes in C57MG cells and can be used to attach the cells to plastic. The availability of a cell-free, biologically active form of Wnt protein might be used to seek Wnt receptors and genes that encode them.

Transgenic mice carrying a *Wnt-1* gene activated by an MMTV LTR exhibit marked epithelial hyperplasia in the mammary glands in both male and female mice, and the females have a high incidence of mammary carcinoma indistinguishable from the virus-induced disease. In addition, some males have mammary carcinomas, and a few transgenic animals have developed salivary gland carcinomas. The transgenic mice document the oncogenic potential of *Wnt-1*, but they also indicate that this single oncogene is not sufficient for tumorigenesis: Only a few cells appear to develop into mammary carcinomas after several months of age, despite the diffuse hyperplasia of mammary tissue. We are therefore looking for cellular genes that might collaborate with *Wnt-1* during tumorigenesis.

By crossing our *Wnt-1* transgenic mice with transgenic mice that carry the *int-2* gene under control of the MMTV LTR, we observed a marked acceleration of tumorigenesis in bitransgenic mice compared to the *Wnt-1* transgenics. Another approach also illustrates the collaboration of *Wnt-1* and *int-2*: Greg Shackleford and Helen Kwan infected *Wnt-1* transgenic mice with MMTV; mammary tumors appeared at least one to two months earlier in virgin or breeding females after MMTV infection than in control animals, and most of the tumors had new MMTV proviruses in a pattern

that implies clonal growth of an infected cell. Nearly half of these tumors had proviral insertions in *int-2* or in *hst* (an adjacent gene that also belongs to the FGF gene family).

Linda Yuschenkoff and Lucy Godley are performing additional crosses between our *Wnt-1* transgenic animals and animals with interesting transgenes expressed in the mammary gland (*int-3*) and animals with targeted mutations in tumor suppressor genes (e.g., the *p53* gene and *Rb* gene, both implicated in human breast cancer).

In collaboration with Larry Donehower, we have found that tumors arise much earlier in the absence of a p53 gene. Lucy is also using differential cDNA cloning to identify genes that may influence conversions from hyperplastic to neoplastic to metastatic phases of carcinogenesis, and making transgenic mice that express wild-type and mutant *p53* genes in the mammary gland.

As an alternative approach to the function of the *Wnt* gene family, we have been collaborating with Cynthia Kenyon's laboratory to study the two nematode homologs of mammalian and insect *Wnt* genes. Both *Ce-Wnt* genes have been sequenced (by Lily Shiue, Greg Shackleford, and Supriya Shivakumar) and the predicted proteins show about 35% amino acid identity with other Wnt proteins, with nearly complete conservation of cysteine residues. Supriya has identified a psoralen-induced mutant of *C. elegans-Wnt-1* that appears to produce embryonic lethality. She is now testing genomic clones and several derivatives for their ability to rescue the mutant or produce additional phenotypes, and she and Greg Jongeward are seeking more mutants of the two *Ce-Wnt* genes. Antisera have also been raised against *Ce-Wnt* protein (by Supriya and Neil Parkin) and are being used to attempt to localize sites of expression.

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